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Superinduction of IL-6 synthesis in human peritoneal mesothelial cells is related to the induction and stabilization of IL-6 mRNA

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Superinduction of IL-6 synthesis in human peritoneal mesothelial cells is related to the induction and stabilization of IL-6 mRNA. The initiation of peritonitis is accompanied by a massive increase in intraperitoneal levels of IL-6. The source of this cytokine and the mechanism by which its levels are increased so dramatically are unknown. We examined the mechanism of IL-6 secretion by HPMC exposed to the milieu present in the peritoneal cavity during the initiation of inflammation. Exposure of HPMC to spent peritoneal dialysis effluent (PDE) or IL-1β resulted in a time- and dose-dependent increase in IL-6 secretion. After 24 hours the IL-6 release (pg/μg cell protein) was increased from 5.0 ± 0.8 in control cells to 16.0 ± 2.4 and to 83.8 ± 17.4 in HPMC treated with PDE and IL-1β (1000 pg/ml), respectively ($N = 5$, $P < 0.05$). If, however, PDE and IL-1β were combined, then the secretion of IL-6 was synergistically increased to 747.7 ± 349.9 ($P < 0.05$ vs. expected additive value). The same effect was evident when PDE was combined with TNFα or mixed with peritoneal macrophage conditioned medium. These changes were not a reflection of HPMC proliferation as estimated by ^{3}H-thymidine incorporation. The "superinduction" of IL-6 release was associated both with the induction and stabilization of IL-6 mRNA. Competitive PCR demonstrated that the amount of IL-6 mRNA (fM/μg total RNA) was increased from 0.35 ± 0.13 in control cells to 11.66 ± 3.89 and to 10.83 ± 5.17 in HPMC treated with PDE and IL-1β (100 pg/ml), respectively ($N = 5$, $P < 0.05$). The combination of PDE + IL-1β synergistically increased IL-6 mRNA levels to 56.33 ± 8.79 ($P < 0.05$ vs. additive value). RNA stability experiments using actinomycin D revealed that the half life of IL-6 mRNA (hours) was increased from 2.8 hours in control cells to 6.7 and 9.4 in HPMC exposed to PDE and IL-1β, respectively. The combination of PDE together with IL-1β resulted in a prolonged stabilization of IL-6 mRNA with levels remaining constant over the 12 hours of the experiment. These data demonstrate that HPMC IL-6 synthesis can be synergistically amplified in the presence of peritoneal dialysis effluent and PMØ-derived cytokines. The results suggest that the peritoneal mesothelium may be responsible for the dramatic rise in IL-6 levels seen during the initial stages of CAPD peritonitis.

Continuous ambulatory peritoneal dialysis (CAPD) has become an established form of therapy for patients with end-stage renal insufficiency. The successful outcome of CAPD depends largely on the long-term preservation of the peritoneal membrane as a dialyzing organ. The integrity of the peritoneal membrane may, however, be easily jeopardized by peritonitis, which still remains a major complication in patients undergoing CAPD. In severe cases the consequence of repeated episodes of peritonitis may be irreparable damage to the peritoneal membrane, loss of ultrafiltration, and treatment failure [1].

Examination of intraperitoneal levels of inflammatory mediators prior to and during peritonitis have revealed that the intraperitoneal levels of prostaglandins (PGE_2 and 6-keto-$PGF_{1\alpha}$) and cytokines (IL-1, TNFα, IL-6, IL-8, and IFN-γ) are significantly elevated during acute phase of peritonitis and subsequently return to basal values when the inflammatory process subsides [2–9]. Moreover, it has been demonstrated that this rise (specifically in TNFα levels) may even precede the occurrence of clinical signs of infection [5, 10]. Thus the specific time courses of cytokine appearance in peritoneal dialysis effluent may identify different stages of the intraperitoneal inflammatory response.

In 1990 Goldman et al [6] and then several other groups [7, 11–13] observed that the initiation of peritonitis in CAPD patients was accompanied by a massive increase (100- to 10,000-fold) in intraperitoneal levels of interleukin-6 (IL-6). The source of this cytokine, its function within peritoneal cavity, and the mechanism by which its levels increased so dramatically have not been fully identified. The observation that the IL-6 dialysate/serum ratios in peritonitis CAPD patients were much higher than could be predicted on the basis of simple diffusion of IL-6 from circulation to the peritoneal cavity provided evidence that IL-6 might be produced intraperitoneally [14]. This focused attention on peritoneal macrophages (PMØ), which are commonly viewed as the first line of defence against invading microorganisms and as such are believed to be a major source of inflammatory mediators initiating the inflammatory response. It has been demonstrated that PMØ from infection-free CAPD patients release significant quantities of eicosanoids and are capable of generating considerable amounts of cytokines when challenged with *S. epidermidis* isolates [15, 16].

More recently, however, increasing evidence suggests that there is another likely candidate as a potent source of intraperitoneal IL-6. The peritoneal mesothelium has been shown to act not only as an inert lining of the peritoneal cavity, but also to possess the capacity to produce cytokines. It has been demonstrated that cultured human peritoneal mesothelial cells (HPMC) secrete biologically active IL-6, and that the IL-6 synthesis can be markedly augmented in response to pro-inflammatory cytokines IL-1β and TNFα as a result of specific induction of IL-6 mRNA [17].

In this study we aimed to characterize the mechanism of IL-6

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secretion by HPMC at both the protein and mRNA levels in the milieu mimicking that of peritoneal inflammation. Our data demonstrate that HPMC IL-6 synthesis can be synergistically amplified in the environment consisting of spent peritoneal dialysate and PMØ-derived cytokines. This observation indicates that the peritoneal mesothelium may be responsible for the dramatic rise in IL-6 levels seen during the initial stages of peritonitis.

Methods

All chemicals, unless otherwise stated, were purchased from the Sigma Chemical Company (Poole, Dorset, UK). Recombinant human IL-1β was obtained from Advanced Protein Products Ltd (Brierley Hill, UK). Its biological activity (expressed as ED_{50}) was below 0.1 ng/ml as determined by the uptake of thymidine by murine C3H/HeJ thymocytes. Human TNFα was obtained from BASF AG (Ludwigshafen, Germany). Its specific activity was 8×10^7 U/mg protein as assessed in a 48 hour L929 bioassay in the absence of actinomycin D. Human recombinant IL-1 receptor antagonist (IL-1ra) was kindly provided by Dr. P. Scholtz (Schering AG, Berlin, Germany). All cytokine preparations were batched and stored at −70°C and freshly thawed for each experiment. Endotoxin contamination of recombinant material was below 0.7 pg/μg protein as assessed by limulus amoebocyte lysate assay (Kabi Vitrum, Stockholm, Sweden). All tissue culture flasks and multi-well plates were obtained from Falcon (Becton-Dickinson UK Ltd, Oxford, UK).

Isolation and culture of human peritoneal mesothelial cells

Human peritoneal mesothelial cells (HPMC) were obtained from the omental tissue of consenting non-uremic patients undergoing elective abdominal surgery. The cells were isolated and characterized essentially as described previously [18]. The identity of mesothelial cells was confirmed by their uniform cobblestone appearance at confluence, by the presence of surface microvilli, and by the uniform positive staining for cytokeratins 8 and 18. The endothelial origin of the cells cultured was excluded by the lack of staining for the factor VIII-related antigen and by the absence of endothelin-1 secretion [19]. The presence of contaminating macrophages was excluded following assessment of Fc receptor expression (using a red cell rosetting assay that was negative in all mesothelial cell cultures examined). Cells were maintained in the Earle's buffered M 199 culture medium (Sera Lab/JRH Biosciences Ltd, Crawley, UK) supplemented with penicillin (100 U/ml), streptomycin (100 μg/ml), L-glutamine (2 mM) (Gibco BRL, Life Technologies Ltd, Paisley, UK), transferrin (5 μg/ml), insulin (5 μg/ml), hydrocortisone (0.4 μg/ml) (all from Sigma) and 10% vol/vol fetal calf serum (FCS) (Sera Lab Ltd). HPMC cultures were maintained at 37°C in a humidified 5% CO_2 atmosphere.

Cells were passaged using a trypsin:EDTA solution (0.125% wt/vol:0.01% wt/vol). All data presented are from experiments performed with cells from the second passage that had previously been growth arrested as described below. Cells used were from the second passage and not later to minimize the number of senescent cells that appeared from the third passage onwards [17, 18].

Establishment of growth arrested HPMC

After HPMC reached confluence in multi-well plates, the 10% FCS-containing medium was removed and cells were washed three times with the culture medium containing 0.1% vol/vol FCS (rest medium). Then HPMC were incubated in the rest medium for 48 hours prior to stimulation. Previous experiments had demonstrated that at this time point the cells were maintained in a viable (as assessed by the lack of LDH release and constant cellular ATP levels) but non-proliferative state (data not shown). Under these conditions the cells could be maintained for up to 96 hours without any significant loss of viability [17].

Peritoneal dialysis effluent

The samples of peritoneal dialysis effluent (PDE) were obtained from consenting non-infected CAPD patients ($N = 9$) using the following technique. Four hours after intraperitoneal infusion of 2.0 liters of fresh peritoneal dialysis solution (Baxter Healthcare Ltd, Thetford, Norfolk, UK), containing 1.36% wt/vol glucose and no other additives, the spent dialysate was drained into the dialysis bag. Then the effluent was transferred to sterile tubes, immediately centrifuged (4000 × g for 30 min) and filtered through 0.2 μm pore size filter (Minisart, Sartorius Ltd, Epsom, UK) to remove any cellular material. Equal volumes of samples from individual patients were pooled and stored in aliquots at −70°C until required.

Peritoneal macrophage (PMØ) conditioned medium

PMØ were harvested from peritoneal dialysis effluent of infection-free CAPD patients as described in detail elsewhere [15]. More than 95% of the cells remaining adherent to the Petri dishes (Becton-Dickinson UK Ltd) were consistently found to be PMØ as assessed morphologically following staining with the Neat Differential Haematology Stain (Guest Medical Ltd., Sevenoaks, UK). Conditioned media were collected from PMØ cultures following a three hour incubation in the culture medium alone (PMØ-CM) or from PMØ stimulated with a strain of *Staphylococcus epidermidis* (*S. epidermidis* DM), isolated from the peritoneal dialysis effluent of a CAPD patient with peritonitis (*S.epi*/PMØ-CM), as previously described [16].

Induction of IL-6 production by HPMC

HPMC were grown to confluence in 24-well plates and growth arrested as previously described. Then cells were washed three times with the rest medium and incubated for 24 hours at 37°C with either the control medium (containing 0.1% FCS) or pooled PDE, both in the presence or absence of IL-1β. In separate experiments HPMC were exposed to the peritoneal macrophage conditioned-media (PMØ-CM or *S.epi*PMØ-CM) combined with either the control medium or PDE. In the inhibition studies HPMC were pre-treated with the transcription inhibitor actinomycin D (Sigma) for 30 minutes at 37°C prior to stimulation.

At designated time intervals the HPMC supernatants were removed, centrifuged at 12000 × g and stored at −70°C until assayed. The cells were washed twice with phosphate buffered saline, pH 7.3 (PBS Dulbecco's; Gibco BRL, Life Technologies Ltd, Paisley, UK) and solubilized with 0.1 N NaOH. Total cellular protein was then analyzed by the modified Bradford method [20] using bovine serum albumin (Sigma) as the standard. Repeated cell counts revealed that 1 μg of cellular protein was equivalent to $3.76 \pm 0.56 \times 10^5$ cells ($N = 20$). All data for IL-6 production are expressed as pg/μg of cellular protein. In all experiments the PDE (and the respective control medium) was supplemented with heparin to a final concentration of 5 U/ml to avoid the clot

Table 1.

α-actin	F: 5′-GGAGCAATGATCTTGATCTT-3′ R: 5′-TCCTGAGGTACGGGTCCTTCC-3′	(204 bp)	[23]
IL-6	F: 5′-TACATCCTCGACGGCATCTC-3′ R: 5′-GCTACATTTGCCGAAGAGCC-3′	(465 bp)	[24]
IL-6 MIMIC	F: 5′-TACATCCTCGACGGCATCTCGCCCCAGCCACCCATTTGTA-3′ R: 5′-GCTACATTTGCCGAAGAGCCGCTCCCTGCCCATTTCTGTC-3′	(412 bp)	

formation. Incubation of HPMC with recombinant cytokines, PDE or PMØ-conditioned media for up to 48 hours did not reduce cell viability as assessed by the LDH release (data not shown).

IL-6 production measurements

The concentration of IL-6 in HPMC supernatants was measured using a specific sandwich-ELISA as previously described [21]. The specific release of IL-6 from HPMC was calculated following the subtraction of background IL-6 levels detected in either control medium, PDE, PMØ-CM or *S.epi*PMØ-CM from those recorded in the postculture supernatants.

RNA isolation, reverse transcription (RT) and polymerase chain reaction (PCR)

HPMC were grown to confluence in six-well plates and rendered quiescent as previously described. Then cells were washed three times with a rest medium and exposed to either the control medium (containing 0.1% FCS) or PDE and incubated in the presence or absence of IL-1β (100 pg/ml) for up to 24 hours. At defined time periods total cellular RNA was extracted from HPMC following lysis with 4 M guanidine isothiocyanate and centrifugation through 5.7 M caesium chloride/0.1 M EDTA solution [22].

The total RNA was reverse transcribed into cDNA using the random hexamers method as previously described [17, 23]. Briefly, the reaction mixture contained 1 μl random hexamers (100 μM; Pharmacia Biosystems Ltd, Milton Keynes, UK), 5 μl dNTPs (2.5 mM, Gibco BRL), 2 μl 10x PCR buffer (100 mM Tris/HCl, 500 mM KCl, 15 mM $MgCl_2$ and 0.01% wt/vol gelatin), 2 μl dithiothreitol (0.1 M, Gibco BRL), 1 μl RNasin® ribonuclease inhibitor (Promega, Southampton, UK), 1 μl M-MLV™ Superscript reverse transcriptase (200 units; Gibco BRL) and 1 μg of total RNA.

PCR amplification was performed in a total volume of 50 μl (2 μl of reverse transcription product and 48 μl of master mix containing 36.25 μl H_2O, 1.25 μl 5′-primer (20 μM), 1.25 μl 3′-primer (20 μM), 4 μl dNTPs, 5 μl 10x PCR buffer and 0.25 μl *Taq* polymerase (2.5 U, Amplitaq®, Perkin Elmer Ltd, Beaconsfield, UK) using a Perkin Elmer 480 Thermocycler (Perkin Elmer Cetus, Applied Biosystems Ltd, Warrington, UK). The PCR protocol was as follows: first cycle −94°C for three minutes, 55°C for one minute, 72°C for one minute; second to 25th cycles −94°C for 40 seconds, 55°C for one minute, 72°C for one minute. The final cycle was 94°C for 40 seconds and 60°C for 10 minutes. PCR was performed for 23 cycles for α-actin and 25 cycles for IL-6. Preliminary experiments revealed that under these conditions the PCR products were generated during the exponential phase of amplification. One-tenth of the PCR reaction from both test (IL-6) and control (α-actin) products were mixed and separated by flat bed electrophoresis in 3% wt/vol NuSieve GTG™ agarose gels (Flowgen Instruments Ltd, Sittingbourne, UK), stained with ethidium bromide (1 μg/ml, Sigma), visualized using UV transillumination, and photographed. The negatives were scanned using the Model GS670 video densitometer (Bio-Rad Laboratories Ltd, Hemel Hempstead, UK) and the density of the bands compared to those of the housekeeping gene using Molecular Analyst™ 2.0 software (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

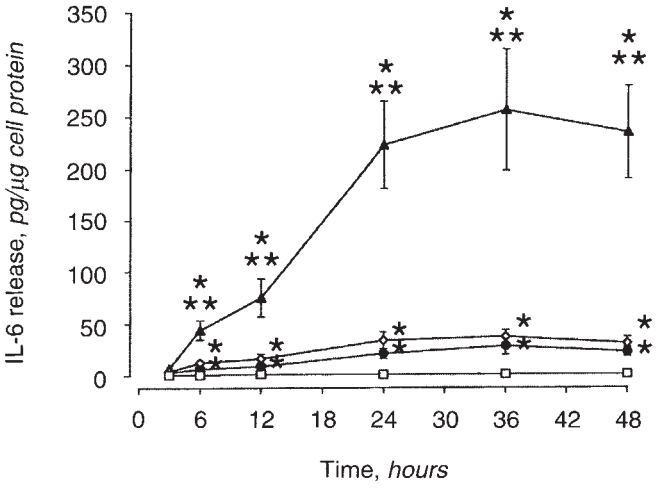


Fig. 1. *Time course of IL-6 synthesis by HPMC in response to PDE and IL-1β.* Growth arrested HPMC were exposed to control medium or PDE in the presence or absence of IL-1β (100 pg/ml) for up to 48 hours. Data represent the mean ± SEM of 7 experiments performed with cells from separate donors. *A statistically significant difference in HPMC IL-6 release compared to the control. **A statistically significant synergistic difference in HPMC IL-6 release compared to the expected additive values for IL-1β and PDE at the same time point. Symbols are: (▲) PDE+IL-1β; (◇) PDE; (●) IL-1β; (□) control.

Competitive PCR

Competitive PCR was performed using the PCR MIMIC™ Construction Kit (Clontech Laboratories Ltd, Cambridge Bioscience, Cambridge, UK). The competitive DNA standard was synthesized by amplification of the *Bam*H I/*Eco*RI fragment of v-*erb*B DNA using composite IL-6 primers. Both the primary PCR amplification with IL-6 composite primers and the secondary PCR amplification with IL-6 specific primers were performed for 30 cycles as follows: 94°C for 45 seconds, 60°C for 45 seconds, 72°C for 90 seconds. The IL-6 MIMIC DNA obtained was purified with

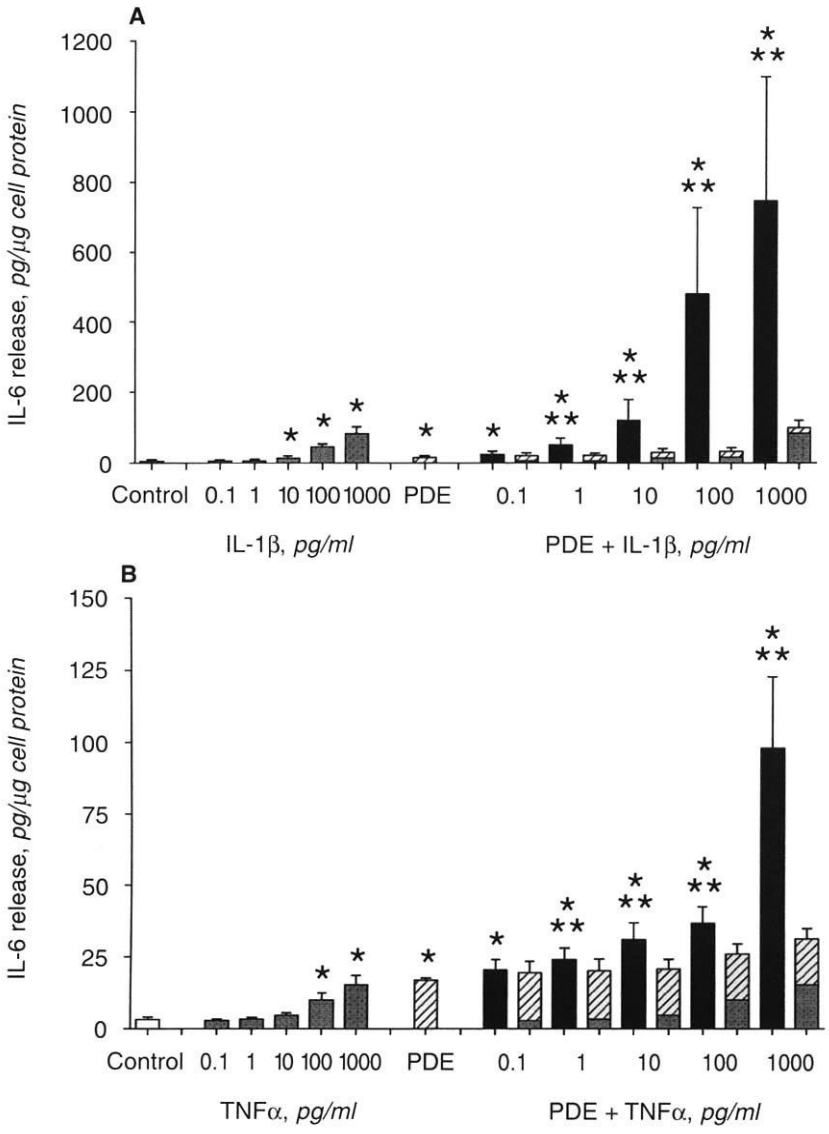


Fig. 2. A *Dose effect of IL-1β on IL-6 generation by HPMC exposed to PDE.* Growth arrested HPMC were exposed to control medium or PDE in the presence of increasing doses of IL-1β (0.1 to 1000 pg/ml) for 24 hours. Data represent the mean ± SEM IL-6 release from 6 experiments with HPMC isolated from different omental specimens. *A statistically significant difference in HPMC IL-6 release compared to the control. **A statistically significant synergistic difference compared to the expected additive values for PDE and IL-1β at the same dose (composite bars). Symbols are: (□) control; (▨) IL-1β; (▨) PDE; (■) IL-1β+PDE. **B.** Dose effect of TNFα on IL-6 generation by HPMC exposed to PDE. Growth arrested HPMC were exposed to control medium or PDE in the presence of increasing doses of TNFα (0.1 to 1000 pg/ml) for 24 hours. Data represent the mean ± SEM IL-6 release from 6 experiments with HPMC isolated from different omental specimens. *A statistically significant difference in HPMC IL-6 release compared to the control. **A statistically significant synergistic difference compared to the expected additive values for PDE and TNFα at the same dose (composite bars). Symbols are: (□) control; (▨) TNFα; (▨) PDE; (■) TNFα+PDE.

Wizard® PCR Preps DNA Purification System (Promega) according to the manufacturer's protocol. The quantity of the purified IL-6 MIMIC product was determined by spectrophotometry at 260 nm. Equal amounts of target cDNA derived from experimental RNA were PCR-amplified in the presence of twofold serial dilutions of the IL-6 MIMIC according to the normal protocol for IL-6 specific primers. The PCR products were resolved on the agarose gels and the optical density of the bands was measured as described previously. Then the ratio of the IL-6 target to the IL-6 MIMIC products was calculated and graphed as a function of the IL-6 MIMIC concentration. From the standard curve generated in this manner the initial amount of IL-6 target cDNA was determined by extrapolating from the point where the amounts of the experimental cDNA and the MIMIC cDNA reached equivalence. After the correction for the difference in size between the IL-6 target and the IL-6 MIMIC products the concentration of mRNA in the original sample was estimated. A representative IL-6-PCR-MIMIC standard curve for a single HPMC extract of mRNA is shown in Figure 8.

Oligonucleotide synthesis

The IL-6 specific oligonucleotide amplification primers and the composite primers for IL-6 MIMIC were synthesized by Genosys Biotechnologies Ltd. (Cambridge, UK). The α-actin specific primers were obtained from Cruachem Ltd. (Glasgow, UK). The primer sequences are in Table 1.

The stability of IL-6 mRNA in HPMC

The inherent stability of IL-6 mRNA in HPMC was assessed by measuring the rate of IL-6 mRNA degradation in cells exposed to the transcription inhibitor actinomycin D. Briefly, growth arrested HPMC were stimulated with either control medium or PDE in the presence or absence of IL-1β (100 pg/ml) for six hours at 37°C and then pulsed with actinomycin D (0.5 μg/ml). At defined times up

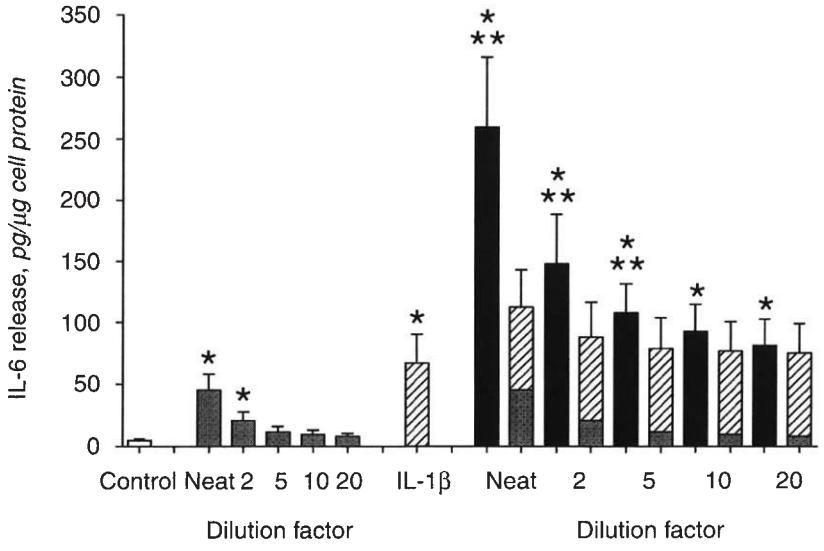


Fig. 3. *Dose effect of PDE on the capacity of HPMC to release IL-6.* Growth arrested HPMC were exposed to increasing dilutions of PDE in the presence or absence of IL-1β (100 pg/ml) for 24 hours. Data represent the mean (± SEM) IL-6 release from 6 experiments with HPMC from separate donors. *A statistically significant difference in HPMC IL-6 release compared to the control. **A statistically significant synergistic difference compared to the expected additive values for IL-1β and equivalent dilution of PDE (composite bars). Symbols are: (□) control; (▨) PDE; (▨) IL-1β; (■) PDE+IL-1β.

to 12 hours after the addition of actinomycin D the total HPMC RNA was extracted, reverse transcribed and PCR amplified as described above. Preliminary experiments revealed that HPMC could be maintained in the presence of 0.5 μg/ml of actinomycin D for up to 12 hours without significant loss of cell viability as assessed by the level of cellular ATP (data not shown).

Effect of interleukin-1 receptor antagonist

In separate experiments growth arrested HPMC were co-incubated with PDE and/or IL-1β in the presence of increasing concentrations of IL-1ra (0.1 to 1000 ng/ml). After 24 hours of exposure the supernatants were assayed for IL-6 and the data normalized for cellular protein content.

Effect of cycloheximide

Growth arrested HPMC were pre-treated with 10 μg/ml of cycloheximide (Aldrich Chemical Company, Gillingham, Dorset, UK) for two hours at 37°C and then stimulated with PDE in the presence or absence of IL-1β (100 pg/ml). After six hours of exposure the total HPMC RNA was extracted, reverse transcribed and PCR amplified as described above.

HPMC proliferation

Proliferation of HPMC in response to PDE was measured by [^{3}H]-thymidine incorporation assay. Cells were plated onto 96-well plates at a density of 1.25×10^4 cells/well, cultured for 24 hours and then growth arrested as previously described. After that subconfluent HPMC were exposed to PDE, pulsed with [^{3}H]-thymidine (as [methyl-^{3}H]-thymidine; 2.5 μCi/ml; specific act. 82 Ci/mM; Amersham International plc, Aylesbury, UK) and incubated in the presence or absence of IL-1β (1000 pg/ml) for 48 hours at 37°C. At the end of the exposure the labeling fluids were removed and cells were washed twice with PBS and precipitated with 10% wt/vol trichloroacetic acid. The precipitate was dissolved in 0.1 N NaOH and the released radioactivity was measured in a beta liquid scintillation counter (LKB Wallac, Turku, Finland).

Statistical analysis

All data were derived from experiments with HPMC prepared from at least five separate donors. All statistical analyses were performed using the Wilcoxon matched-pairs signed ranks test for non-parametric data (Statview® SE; Abacus Concepts Inc., Berkeley, CA, USA) with a P value of less than 0.05 being considered as significant. All data are presented as mean (± SEM).

Results

Induction of HPMC IL-6 production

Incubation of growth arrested HPMC with PDE resulted in a time-dependent increase in IL-6 generation (Fig. 1). The time course and the magnitude of the IL-6 release was comparable to that induced by 100 pg/ml IL-1β. In each case the secretion of IL-6 became significant above the control level after six hours and reached a plateau by 24 hours. The combination of PDE together with IL-1β, however, induced an IL-6 production above the levels generated by each stimulus alone, and significantly above the predicted additive value. This synergistic increase in the HPMC IL-6 synthesis was first discernible at six hours, followed by large increments over the next 18 hours and a plateau by 24 hours. Repeated measurements over the whole study revealed that in HPMC from different donors the increase in 24-hour IL-6 release in response to a combination of PDE and 100 pg/ml IL-1β ranged from 1.1- to 27.2-fold above expected additive levels (median value 3.0-fold, $N = 40$).

The synergistic effect of the combined PDE and IL-1β stimulation was dependent on the dose of IL-1β (Fig. 2A). It became significant above additive values when PDE was combined with 1 pg/ml IL-1β and maximal synergy was obtained at the highest IL-1β dose tested (1000 pg/ml) when the 24-hour HPMC IL-6 secretion was 747.74 ± 349.93 pg/μg cell protein compared to the additive value of 99.81 ± 19.16 pg/μg cell protein ($N = 6$, $z = 2.201$, $P < 0.027$). The same effect was evident when IL-1β was replaced with TNFα, although the degree of stimulation was much less pronounced (Fig. 2B). A 24-hour exposure of HPMC to 1000

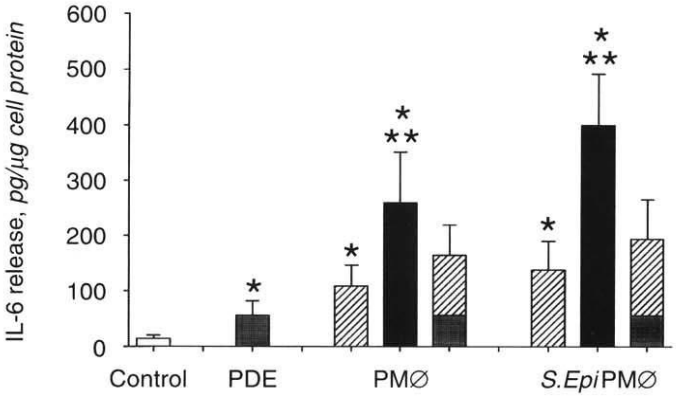


Fig. 4. *Effect of macrophage-conditioned media and PDE on HPMC IL-6 synthesis.* IL-6 generation by HPMC was measured following a 24-hour exposure to PMØ-CM or *S. Epi* PMØ-CM mixed (1:5) with either control medium or PDE. Data represent the mean ± SEM IL-6 release from 6 experiments with cells prepared from separate omental specimens. Symbols are: (□) control; (▨) PDE; (▨) macrophage-CM; (■) PDE + macrophage-CM. *A statistically significant difference in HPMC IL-6 release compared to the control. **A statistically significant synergistic difference in HPMC IL-6 release compared to the expected additive values for macrophage supernatants and PDE (composite bars).

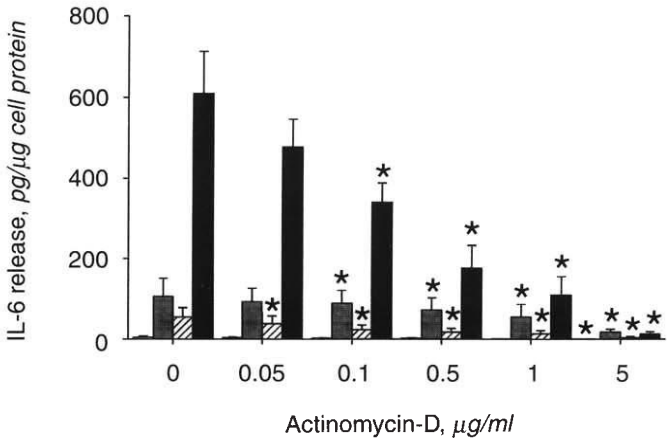


Fig. 5. *Actinomycin D inhibition of IL-6 release from HPMC exposed to PDE and IL-1β.* Growth arrested HPMC were pre-treated with increasing concentrations of actinomycin D for 30 minutes and then exposed to control medium or PDE in the presence or absence of IL-1β (100 pg/ml) for 24 hours. Data presented are the mean ± SEM of 5 experiments with HPMC isolated from different omental specimens. *A statistically significant difference compared to untreated controls. Symbols are: (□) control; (▨) IL-1β; (▨) PDE; (■) PDE+IL-1β.

pg/ml TNFα resulted in the IL-6 release of 15.17 ± 3.31 pg/μg cell protein ($N = 6$, $z = 2.201$, $P < 0.027$). The same dose of TNFα applied together with PDE generated the IL-6 secretion of 98.12 ± 24.33 pg/μg cell protein compared to the expected additive value of 31.88 ± 2.96 pg/μg cell protein ($N = 6$, $z = 2.201$, $P < 0.027$).

The synergistic effect of the PDE and IL-1β combination was also related to the PDE concentration (Fig. 3). Progressive dilutions of the PDE reduced its capacity to stimulate HPMC IL-6 secretion. The potential of the PDE to superinduce IL-6 synthesis in the presence IL-1β, however, still occurred at dilutions between 1:2 and 1:5.

Induction of HPMC IL-6 by PMØ conditioned medium

The mean concentrations of IL-6 in undiluted pooled peritoneal macrophage-conditioned medium (PMØ-CM or *S.epi* PMØ-CM) were 72 and 161 pg/ml, respectively. These values were subtracted from those detected in the HPMC supernatants to assess specific release of mesothelial cell IL-6.

Exposure of HPMC to the peritoneal macrophage-conditioned medium obtained either from control cultures (PMØ-CM) or following the stimulation with *S.epidermidis* (*S.epi*PMØ-CM) resulted in a time- and dose-dependent stimulation of HPMC IL-6 secretion. At equivalent dilutions a 24-hour IL-6 release triggered by *S.epi*PMØ-CM always exceeded that induced by PMØ-CM. At the optimal dilution (1:5) the recorded IL-6 levels were 108.60 ± 36.20 and 137.93 ± 50.38 pg/μg cell protein for PMØ-CM and *S.epi*PMØ-CM, respectively, ($N = 6$, $z = 2.201$, $P < 0.027$ vs. control: 13.95 ± 4.30 pg/μg cell protein). The similar pattern of IL-6 secretion was detected when HPMC were exposed to PMØ-conditioned media mixed with PDE. In this case, however, the degree of stimulation was significantly higher when compared to HPMC treated with equivalent dilutions of PMØ-CM in control medium (Fig. 4). At the 1:5 dilution with PDE the levels of HPMC-derived IL-6 were 259.59 ± 89.23 and 400.59 ± 88.94 pg/μg cell protein for PMØ-CM and *S.epi*PMØ-CM, respectively ($N = 6$, $z = 2.201$, $P < 0.027$ vs. PDE alone: 55.73 ± 23.03 pg/μg cell protein). These levels were significantly above the calculated sum of individual effects of respective PMØ supernatants and spent dialysate.

Actinomycin D inhibition

Pre-exposure of HPMC to actinomycin D for 30 minutes at 37°C resulted in a dose-dependent inhibition of both PDE- and IL-1β-stimulated IL-6 release (Fig. 5). The maximal inhibition was obtained with the highest dose tested (5 μg/ml) when the IL-6 generation was reduced by means of 83.4 ± 6.9%, 92.0 ± 4.5%, and 97.9 ± 1.0% for HPMC exposed to IL-1β, PDE, and a combination of IL-1β with PDE, respectively ($N = 5$, $z = 2.023$, $P < 0.05$ for all). Under the same conditions the constitutive IL-6 release was decreased by 94.3 ± 4.8% ($N = 5$, $z = 2.023$, $P < 0.05$).

Effect of interleukin-1 receptor antagonist

In HPMC treated with either control medium or PDE, both in the presence or absence of IL-1β (100 pg/ml), the simultaneous addition of IL-1ra resulted in a dose-dependent reduction in the HPMC IL-6 generation (Fig. 6). Following a 24-hour co-incubation with the highest concentration of IL-1ra added (1000 ng/ml) HPMC IL-6 synthesis was reduced by means of 85.0 ± 9.4% and 81.8 ± 10.3% in control cells and HPMC exposed to PDE, respectively ($N = 5$, $z = 2.023$, $P < 0.05$ for both). Under the same conditions the production of IL-6 was reduced by 92.7 ± 5.7% and 81.9 ± 14.5% in cells treated with IL-1β and a combination of PDE+IL-1β, respectively ($N = 5$, $z = 2.023$, $P < 0.05$ for both).

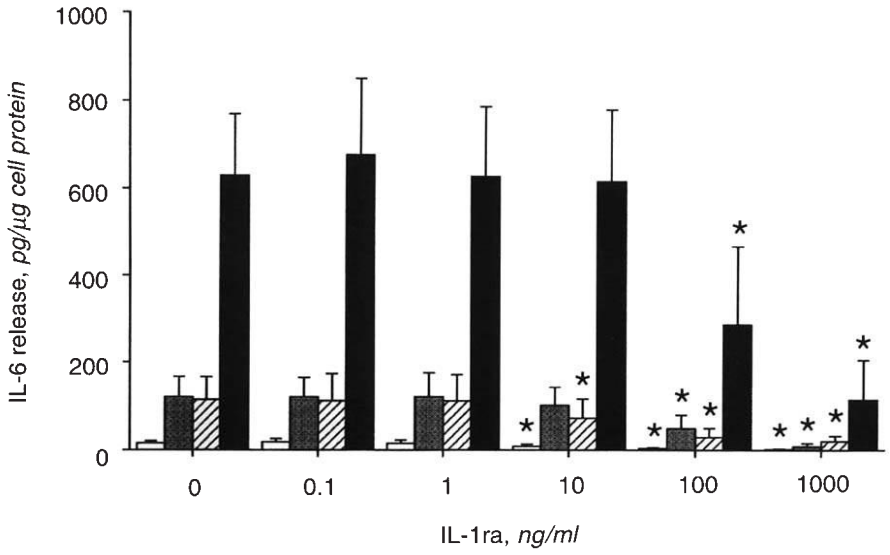


Fig. 6. *Dose effect of IL-1ra on IL-6 generation by HPMC exposed to PDE and IL-1β.* Growth arrested HPMC were exposed to control medium or PDE in the presence or absence of IL-1β (100 pg/ml), and simultaneously treated with increasing concentrations of IL-1ra (0.1 to 1000 ng/ml) for 24 hours. Data represent the mean ± SEM of 5 experiments with HPMC isolated from different omental specimens. *A statistically significant difference compared to untreated controls. Symbols are: (□) control; (▨) IL-1β; (▨) PDE; (■) PDE + IL-1β.

PCR analysis of HPMC IL-6 gene expression

For all mesothelial cell lines examined, the PCR amplification of the reverse transcribed HPMC RNA, using specific IL-6 primers, produced single band IL-6 transcripts of 465 base pairs. The IL-6 mRNA expression was assessed by comparison with the 204 base pair transcripts of the α-actin housekeeping gene from the same samples. Incubation of HPMC in the presence of either PDE or IL-1β (100 pg/ml) resulted in an increased expression of IL-6 mRNA as compared to unstimulated controls (Fig. 7). The combination of PDE and IL-1β generated a prolonged increase in the expression of IL-6 mRNA and above the levels recorded in response to PDE or IL-1β alone. The maximal IL-6 mRNA expression was observed 4 to 6 hours after the addition of IL-1β and/or PDE.

Competitive PCR

The conventional PCR amplification with the use of an endogenous internal standard (α-actin) gives only an estimate of relative changes in mRNA expression. We therefore developed a semi-quantitative competitive MIMIC-PCR to determine changes in IL-6 mRNA abundance more precisely (Fig. 8). The coefficient of variation in these assays varied between 62% to 107% depending on stimulation ($N = 20$ determinations).

After six hours of incubation the amount of IL-6 mRNA (fM/μg of total cellular RNA) was increased from 0.35 ± 0.13 in control cells to 10.83 ± 5.17 in HPMC treated with IL-1β (100 pg/ml) and to 11.66 ± 3.89 in HPMC exposed to PDE ($N = 5$, $z = 2.023$, $P < 0.05$ for both; Fig. 9). Combined stimulation of HPMC with PDE and IL-1β resulted in the IL-6 mRNA generation of 56.33 ± 8.79 fM/μg of total cellular RNA ($N = 5$, $z = 2.023$, $P < 0.05$); this level was significantly above the calculated sum of individual stimulatory capacities of PDE and IL-1β (250.5 ± 39.1% of the predicted additive value; $N = 5$, $z = 2.023$, $P < 0.05$).

Stability of IL-6 mRNA in HPMC

The stability of the PDE- and cytokine-induced HPMC IL-6 mRNA was assessed in actinomycin D chase experiments. These experiments revealed that in cells treated singly with PDE or IL-1β (100 pg/ml) the degradation of IL-6 mRNA was delayed compared to unstimulated controls (Fig. 10). The calculated half-life of IL-6 mRNA was extended from 2.8 hours in control cells to 9.4 and 6.7 hours in HPMC exposed to IL-1β and PDE, respectively. The combination of PDE+IL-1β, however, resulted in prolonged stabilization of IL-6 mRNA with levels remaining constant over the 12 hours of the experiment.

Effect of cycloheximide

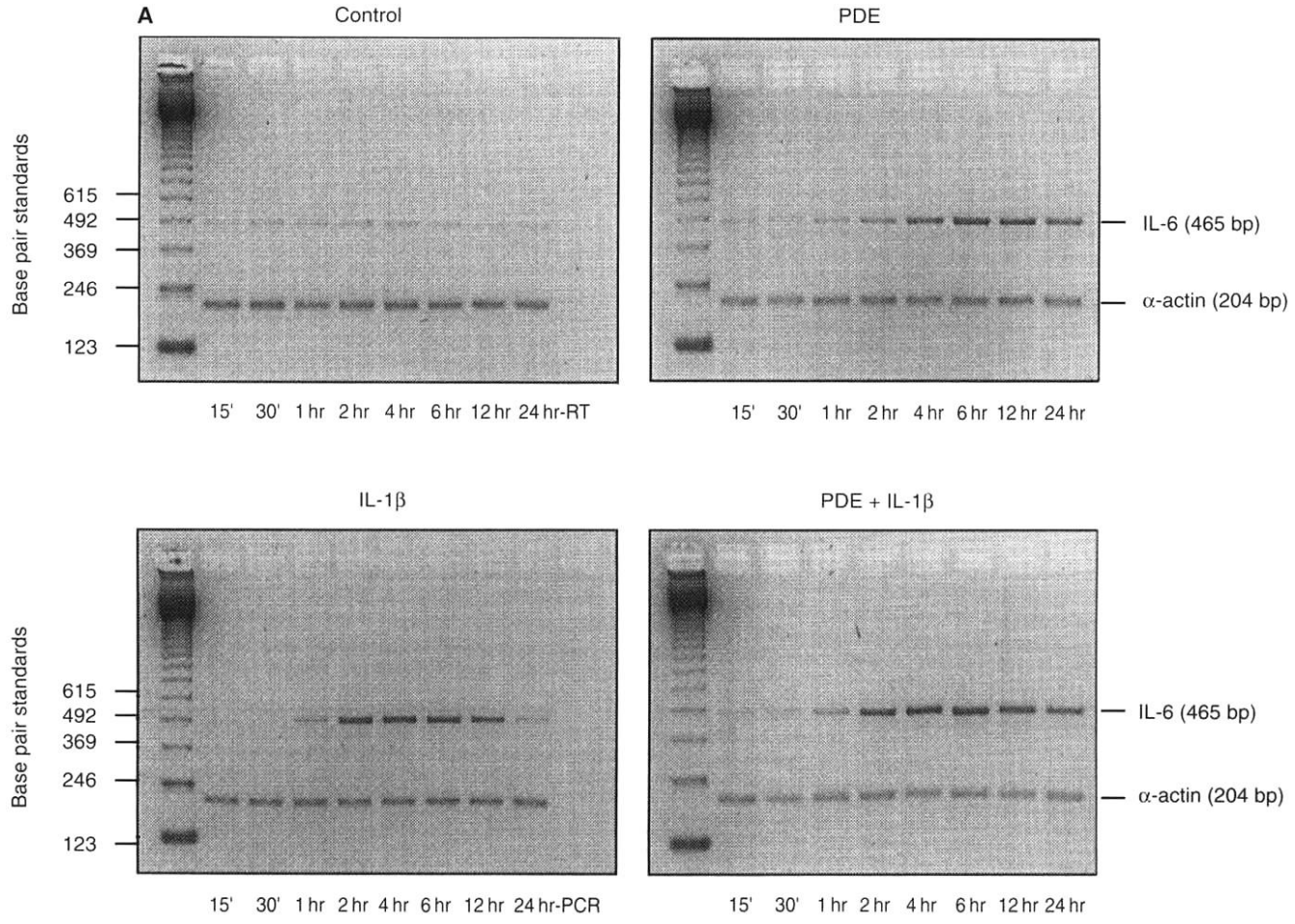
Pre-exposure of HPMC to cycloheximide resulted in 1.3-fold and 2.2-fold increase in IL-6 mRNA expression (as measured by IL-6/α-actin O.D. ratio) in response to IL-1β or PDE, respectively. Moreover, cycloheximide promoted 5.4-fold increase in IL-6 mRNA expression in control cells in the absence of additional stimulation. In contrast, cycloheximide did not appear to increase the IL-6 mRNA expression in cells treated with PDE+IL-1β. In this case the IL-6/α-actin O.D. ratio was 0.79 and 0.75 for HPMC pre-exposed to cycloheximide and vehicle only, respectively.

HPMC proliferation

Exposure of subconfluent HPMC to PDE increased the cellular incorporation of [^{3}H]-thymidine in a time- and dose-dependent manner. Following a 48-hour incubation the increase in [^{3}H]-thymidine incorporation was observed in HPMC exposed to PDE dilutions as low as 1:128. The peak stimulation was achieved with a 1:2 PDE dilution where [^{3}H]-thymidine incorporation (cpm) was 2709 ± 904 compared to 1029 ± 368 in controls ($N = 6$, $z = 2.201$, $P < 0.027$). The addition of IL-1β did not modify the [^{3}H]-thymidine incorporation into HPMC treated with either control medium or PDE. In HPMC incubated with PDE (1:2) in the presence of IL-1β (1000 pg/ml) the incorporation was 2239 ± 645 cpm ($N = 6$, $z = 0.524$, *NS*).

Discussion

The demonstration that HPMC secrete IL-6 *in vitro* has led to the suggestion that the increase in the intraperitoneal levels of IL-6 occurring during peritonitis might be, at least in part, of mesothelial origin [17]. To assess the potential of the mesothelium to contribute to this rise more precisely, in the present study we



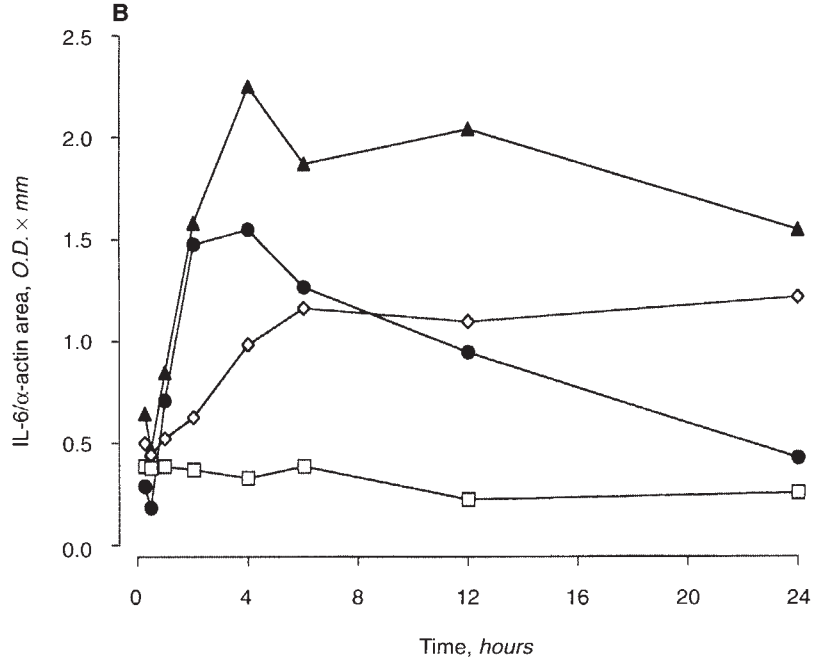


Fig. 7. *IL-6 mRNA expression in HPMC treated with PDE and IL-1β.* Following timed exposure of growth arrested HPMC to control medium or PDE in the presence or absence of IL-1β (100 pg/ml) total cellular RNA was extracted, reverse transcribed and PCR amplified for IL-6 and α-actin. PCR products were separated by electrophoresis in ethidium bromide stained 3% agarose gels (**A**), and the results were presented as the densitometric ratios of IL-6/α-actin (**B**). Symbols are: (▲) PDE + IL-1β; (◇) PDE; (●) IL-1β; (□) control.

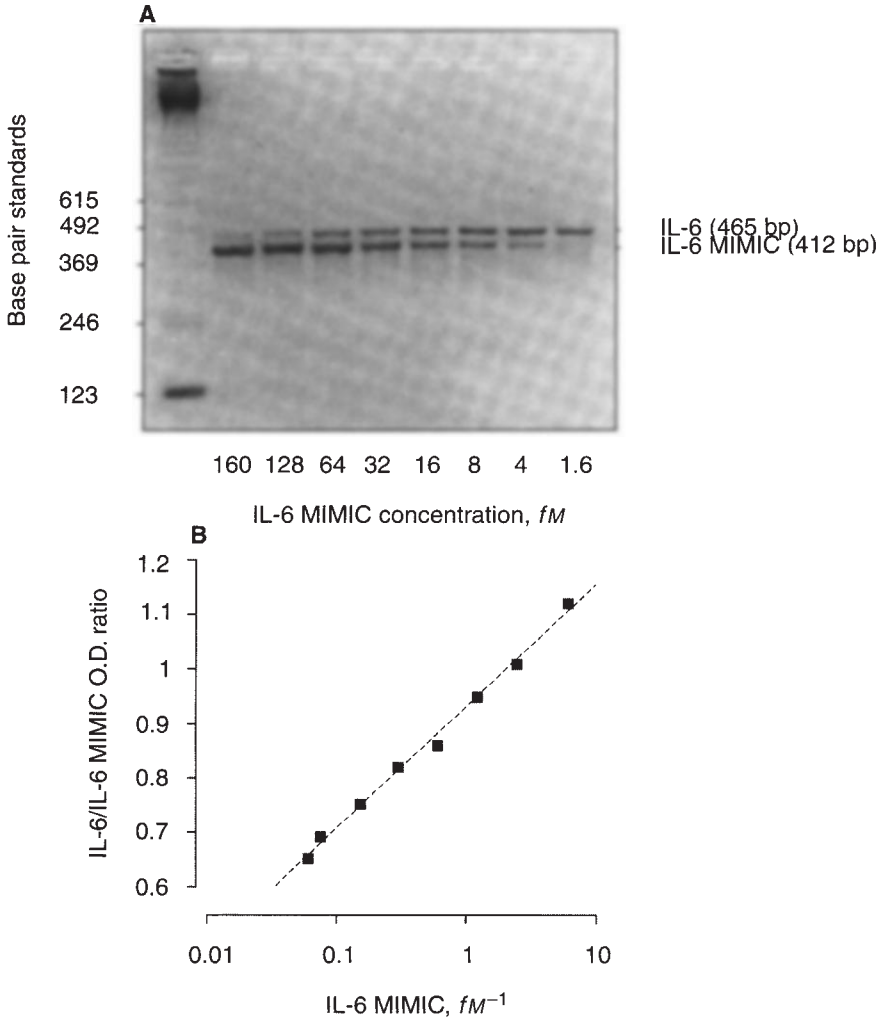


Fig. 8. *Representative competitive PCR standard curve for a single extract of HPMC RNA.* The experimental HPMC RNA was reverse transcribed and the constant amounts of sample target cDNA were subjected to PCR amplification in the presence of serial dilutions of competitive DNA standard (IL-6 MIMIC). PCR products were resolved on 3% agarose/ethidium bromide gel (**A**). Following densitometric scanning the IL-6/IL-6 MIMIC O.D. ratio was plotted as a function of IL-6 MIMIC concentration and the amount of target cDNA determined from the point in which the ratio reached 1:1 (**B**). $y = 0.225 \log(x) + 0.931$.

have investigated the induction of IL-6 synthesis in HPMC exposed to peritoneal dialysis effluent.

Incubation of growth arrested HPMC in the presence of four-hour spent dialysate pooled from infection-free CAPD patients resulted in a time- and dose-dependent increase in the release of IL-6. PCR amplification of the reverse transcribed HPMC RNA revealed that this increase was accompanied by the induction of IL-6 mRNA. In this model the release of IL-6 in response to PDE may represent the mesothelial fraction of low IL-6 levels detected normally in drained dialysates from non-infected CAPD patients.

To mimic the inflammatory state *in vivo*, PDE has further been supplemented with IL-1β and TNFα, the potent pro-inflammatory cytokines known to be present in the peritoneum during initial stages of infection [4, 5, 9]. The effect of these cytokines on the IL-6 release by HPMC *in vitro* has previously been well established [17]. Both IL-1β and TNFα have been shown to induce expression of specific IL-6 mRNA and increase the secretion of IL-6 from HPMC. Interestingly, however, the combination of these cytokines produced only an additive release of HPMC IL-6, contrasting with the synergistic effects seen in many systems tested [19, 25, 26]. The present study demonstrates that in the environment of PDE the stimulatory action of IL-1β, and to lesser extent TNFα, can be amplified in a synergistic fashion. Combination of IL-1β together with PDE resulted in a synergistic increase in IL-6 synthesis that was significantly greater than the additive values of the two stimuli alone. This synergistic effect appeared to depend on both the concentration of PDE and the dose of cytokine, and occurred well within the range of IL-1β concentrations present in the peritoneal cavity during peritonitis [4, 9].

The similar rise in HPMC IL-6 release was achieved when recombinant cytokines were replaced with PMØ-conditioned medium. The supernatants from both control and *S.epidermidis*-stimulated PMØ induced a synergistic increase in HPMC IL-6 release when combined with PDE. Previous studies, utilizing anti-cytokine antibodies and specific cytokine inhibitors, have clearly identified that the stimulatory capacity of PMØ-conditioned media towards HPMC IL-6, IL-8, and prostaglandin synthesis was primarily related to their IL-1β and TNFα content [27, 28]. Indeed, co-incubation of HPMC with IL-1ra significantly reduced the PDE+IL-1β–stimulated IL-6 release, indicating that IL-1β is a key mediator in the superinduction phenomenon. In this respect Fieren et al have demonstrated that PMØ harvested

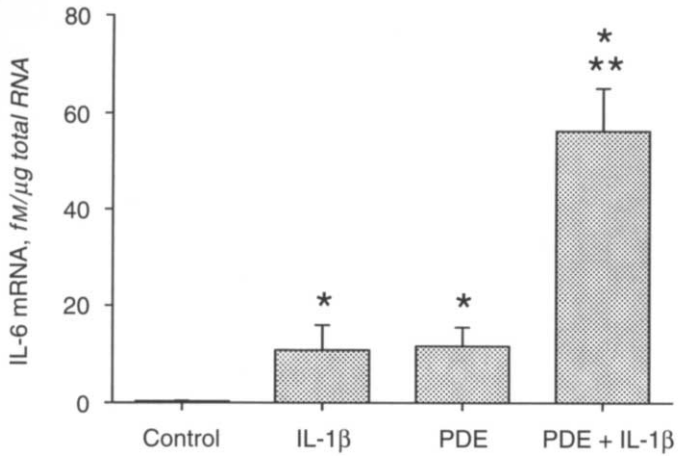


Fig. 9. *Quantitation of IL-6 mRNA in HPMC exposed to PDE and IL-1β.* Growth arrested HPMC were exposed to either control medium or PDE in the presence or absence of IL-1β (100 pg/ml) for six hours. At this time point total RNA was extracted, reverse transcribed and the cellular IL-6 mRNA concentration was assessed by competitive PCR. Data presented are the mean ± SEM amount of IL-6 mRNA (fM/μg of total extracted RNA) in HPMC from 5 separate donors. *A statistically significant difference in the amount of HPMC IL-6 mRNA compared to the control. **A statistically significant synergistic difference in HPMC IL-6 mRNA compared to the expected additive value for PDE and IL-1β.

from CAPD patients during episodes of peritonitis display an increased capacity to secrete IL-1β upon LPS stimulation [29]. Interestingly, IL-1ra also appeared to be capable of decreasing the constitutive IL-6 release, suggesting that this might in part be due to the inhibition of endogenously secreted mesothelial IL-1β [30]. The synergistic IL-6 increase does not seem, however, to be attributable to enhanced HPMC proliferation. Although PDE stimulated [^{3}H]-thymidine incorporation into HPMC, this effect was not modified by the presence of IL-1β.

Pre-treatment of HPMC with actinomycin D resulted in a dose-dependent inhibition of PDE+IL-1β–stimulated IL-6 release, indicating that this effect is regulated at the transcriptional level. We demonstrated that HPMC responded to a combination of PDE and IL-1β by dramatically increasing IL-6 mRNA levels. Quantitative analysis by competitive RT/PCR revealed the synergistic nature of this increase, since the total amount of IL-6 mRNA generated under these conditions was significantly above the predicted additive value.

The IL-6 gene expression in HPMC treated with a combination of PDE and IL-1β appeared also to be regulated at the post-transcriptional level as indicated by actinomycin D chase experiments. The IL-6 mRNA transcript induced in response to PDE+IL-1β was much more stable than that induced by either PDE or IL-1β alone. The mechanism underlying enhanced IL-6 mRNA stability may involve the inhibition of mRNA-destabilizing proteins or cell-type specific processing of mRNA sequences recognized by these proteins. Stabilization of mRNA is an effective mechanism for increasing the pool of translatable mRNA and ultimately the amount of protein synthesized. Thus the combination of increased gene transcription together with the prolonged stability of the mRNA transcripts may account for the superinduction of IL-6 synthesis in HPMC exposed to PDE in the presence of IL-1β.

Transcriptional activation of IL-6 mRNA by PDE+IL-1β appeared to be accomplished without the need for newly synthesized protein intermediates, since pre-treatment of HPMC with cycloheximide had no apparent effect on the PDE+IL-1β–induced IL-6 mRNA. In contrast, cycloheximide increased the expression of IL-6 mRNA both in cells treated singly with PDE or IL-1β and also in unstimulated controls. Cycloheximide has previously been shown to enhance IL-6 mRNA expression in some cell types [31] presumably by inhibiting the synthesis of mRNA-destabilizing proteins and/or transcriptional repressors. The complete stabilization of steady-state IL-6 mRNA levels by the combination of PDE together with IL-1β might explain the inability of cycloheximide to further augment the IL-6 mRNA expression. The more precise characterization of the mechanisms governing the IL-6 gene expression in HPMC should clarify this issue. The transcriptional control elements identified in the 5′-flanking region of the IL-6 gene include glucocorticoid responsive element (GRE), cAMP responsive element (CRE), serum responsive element (SRE), and the NF-κB, NF-IL-6 and AP-1 binding sites [32]. The potential involvement of these elements in the PDE+IL-1β–induced HPMC IL-6 gene expression is currently being investigated.

The nature of the PDE stimulatory potential remains hypothetical. Following a four-hour dwell the peritoneal dialysis effluent contains a plethora of "uremic molecules" as well as a mixture of different mediators of both systemic and local origin. Some of these mediators, including LTB_4, TNFα, TGF-β, and IL-4, have been shown to synergize with IL-1 in the production of IL-6 in other cell systems [25, 31, 33–36]. Whether the PDE-mediated mesothelial IL-6 superinduction is related specifically to the local activation within the peritoneum or related to the uremic state *per se* remains to be determined.

The function of intraperitoneal IL-6 is unknown. IL-6 is a pleiotropic cytokine involved in immune and inflammatory responses. Multiple actions of IL-6 include induction of B-lymphocyte differentiation and immunoglobulin production, activation and differentiation of T-cells, and stimulation of hepatic synthesis of acute phase proteins [37]. Increased levels of IL-6 have been associated with several inflammatory conditions including septic shock, rheumatoid arthritis, and meningitis [32]. IL-6 may also exhibit anti-inflammatory properties since it is capable of suppressing the expression of IL-1 and TNFα [38, 39], and inducing circulating IL-1ra and soluble TNFp55 receptor [40]. This led to the suggestion that the intraperitoneal IL-6 might act to control rather than to amplify the inflammatory response [41]. The demonstration that the peritoneal mesothelium is able to produce massive amounts of IL-6 during peritonitis adds further evidence to the belief that the mesothelial cell plays a central role in the control of inflammation in the peritoneal cavity.

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Appendix

Abbreviations are: CAPD, continuous ambulatory peritoneal dialysis; HPMC, human peritoneal mesothelial cell; IL-1β, interleukin-1β; IL-1ra, interleukin-1 receptor antagonist; IL-6, interleukin-6; IL-8, interleukin-8; PDE, peritoneal dialysis effluent; PMØ, peritoneal macrophage; PMØ-CM, peritoneal macrophage conditioned medium; *S.epi*PMØ-CM, *S. epidirmidis*-stimulated PMØ conditioned medium; RT/PCR, reverse transcription/polymerase chain reaction; TNFα, tumor necrosis factor-α.

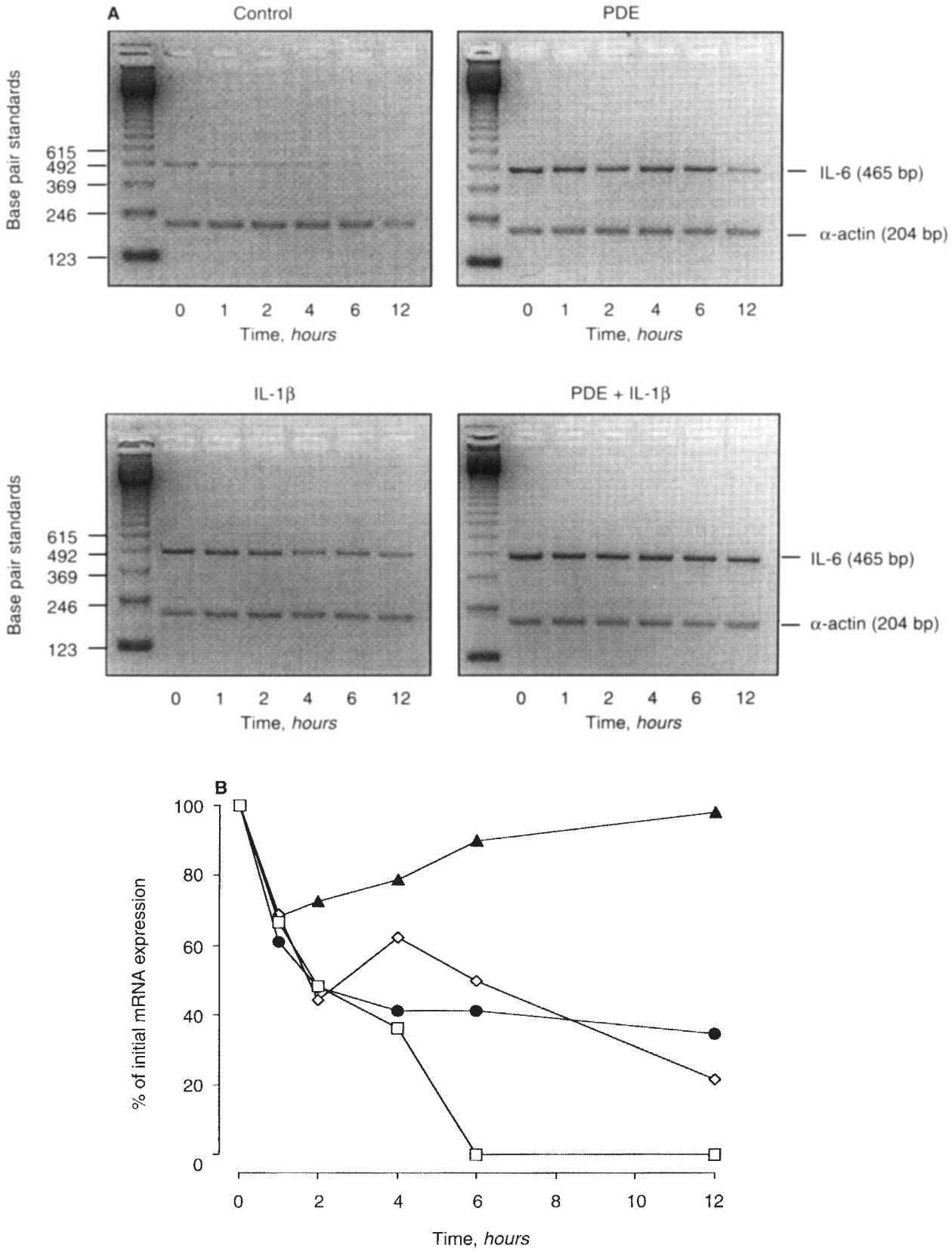

Fig. 10. *Stability of IL-6 mRNA in HPMC treated with PDE and IL-1β.* Growth arrested HPMC were stimulated with either control medium or PDE in the presence or absence of IL-1β (100 pg/ml) for six hours and then pulsed with 0.5 μg/ml of actinomycin D (time zero). The rate of mRNA degradation was determined by extracting total HPMC RNA at various times after the addition of actinomycin D, and performing RT/PCR. PCR products were separated by flat bed electrophoresis in ethidium bromide stained 3% agarose gels (**A**). Following densitometric analysis the IL-6/α-actin O.D. ratios were calculated and the results were expressed as a percentage of the value obtained at time zero (**B**). Symbols are: (▲) PDE + IL-1β; (●) IL-1β; (◇) PDE; (□) control.

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